

La Crosse Bunyavirus Nonstructural Protein NSs Serves To Suppress the Type I Interferon System of Mammalian Hosts[▽]

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La Crosse virus (LACV) is a mosquito-transmitted member of the *Bunyaviridae* family that causes severe encephalitis in children. For the LACV nonstructural protein NSs, previous overexpression studies with mammalian cells had suggested two different functions, namely induction of apoptosis and inhibition of RNA interference (RNAi). Here, we demonstrate that mosquito cells persistently infected with LACV do not undergo apoptosis and mount a specific RNAi response. Recombinant viruses that either express (rLACV) or lack (rLACVΔNSs) the NSs gene similarly persisted and were prone to the RNAi-mediated resistance to superinfection. Furthermore, in mosquito cells overexpressed LACV NSs was unable to inhibit RNAi against Semliki Forest virus. In mammalian cells, however, the rLACVΔNSs mutant virus strongly activated the antiviral type I interferon (IFN) system, whereas rLACV as well as overexpressed NSs suppressed IFN induction. Consequently, rLACVΔNSs was attenuated in IFN-competent mouse embryo fibroblasts and animals but not in systems lacking the type I IFN receptor. In situ analyses of mouse brains demonstrated that wild-type and mutant LACV mainly infect neuronal cells and that NSs is able to suppress IFN induction in the central nervous system. Thus, our data suggest little relevance of the NSs-induced apoptosis or RNAi inhibition for growth or pathogenesis of LACV in the mammalian host and indicate that NSs has no function in the insect vector. Since deletion of the viral NSs gene can be fully complemented by inactivation of the host's IFN system, we propose that the major biological function of NSs is suppression of the mammalian innate immune response.

La Crosse virus (LACV) is an important mosquito-borne pathogen in North America, causing severe encephalitis and aseptic meningitis in children and young adults (37, 42, 57). Around 75 to 100 cases of La Crosse encephalitis requiring hospitalization are reported annually (12), and up to 57% percent of these patients need to be admitted to the intensive care unit (37). More than 10% of the hospitalized patients will have long-lasting neurological deficits (37, 38), with severe economic and social consequences (52). As less than 1.5% of LACV infections are clinically apparent, it is estimated that more than 300,000 infections occur annually in the Midwestern United States alone (11, 38).

Like other arboviruses, LACV cycles between vertebrate and invertebrate hosts, able to replicate both in mammals and in insects. Depending on the host, however, the outcome of infection is different (7). In mammalian cells, infection is lytic and causes host cell shutoff and cell death. In insect cells,

infection is noncytolytic and leads to long-term viral persistence.

LACV belongs to the California serogroup of the genus *Orthobunyavirus*, family *Bunyaviridae* (7). Bunyaviruses are a large group of mainly arthropod-transmitted viruses. They are classified into five genera: *Orthobunyavirus*, *Phlebovirus*, *Hanta-virus*, *Nairovirus*, and *Tospovirus*. Some members are human pathogens and can cause encephalitis, febrile illness, or hemorrhagic fever; among them are LACV, Oropouche virus, Hantaan virus, Rift Valley fever virus (RVFV), and Crimean-Congo hemorrhagic fever virus (19, 56). Bunyaviruses are enveloped and have a trisegmented single-stranded RNA genome of negative or ambisense polarity, replicate in the cytoplasm, and bud into the Golgi apparatus. They encode four common structural proteins: the viral polymerase (L) on the large (L) segment, two glycoproteins (Gn and Gc) on the medium (M) segment, and the viral nucleocapsid protein (N) on the smallest (S) segment.

Some bunyaviruses encode on their S segment a nonstructural protein which is termed NSs. For LACV, two different functions for viral pathogenicity have been assigned to this accessory protein. Firstly, NSs has similarity to the *Drosophila* protein reaper and is an efficient inducer of apoptosis if overexpressed in mammalian cells (14). Our own studies using recombinant LACV expressing or lacking NSs supported this

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notion (6). Secondly, overexpressed NSs was shown to efficiently suppress RNA interference (RNAi) in mammalian cells (49), suggesting that LACV NSs is important for evasion of this antiviral system. By contrast, in vivo studies with the orthobunyavirus Bunyamwera virus (BUNV) or the phlebovirus RVFV provided evidence that their NSs proteins are efficient inhibitors of type I interferon (IFN- α/β) synthesis and that this activity is relevant for infection of mammalian hosts (8, 25, 55).

IFNs are cytokines which establish a first line of defense against viral pathogens by stimulating the synthesis of antiviral proteins and activating the adaptive immune system (43). In most cell types, virus infection activates a signaling pathway that results in the immediate synthesis of IFN- β and IFN- α_4 , which is followed by a second burst of IFN which comprises a large number (at least 14 in mice) of different α subtypes (36, 53). In plasmacytoid dendritic cells, which are the professional IFN producers of the immune system, IFN- α is directly synthesized after stimulation (13).

The biological function of LACV NSs in the mammalian host has to date not been clarified, and the role of NSs in infection of the insect vector has not been resolved for any of the bunyaviruses. Here, we used our recently generated recombinant LACV mutant lacking NSs (rLACVdelNSs) (6) to investigate these issues. Our results strongly suggest that LACV NSs evolved mainly to counteract the antiviral IFN system of the mammalian host.

MATERIALS AND METHODS

Chemicals, cells, and viruses. Poly(I · C) (Sigma) was dissolved and used as indicated by the manufacturer. Mouse embryo fibroblasts (MEFs) were prepared by trypsin digestion of 14-day-old embryos. The cultures were used between passages 3 to 8. MEFs and human 293 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). BSR-T7/5 cells (10), which constitutively express the bacteriophage T7 RNA polymerase, were additionally provided with 1 mg/ml geneticin (G418). *Aedes albopictus* C6/36 and U4.4 cells (a kind gift of D. T. Brown, North Carolina State University, Raleigh, NC) and *Aedes triseriatus* MAT cells were grown at 30°C (C6/36 and MAT cells) or 28°C (U4.4 cells) without CO₂ in Leibowitz L-15 medium (Gibco-BRL) supplemented with 10% FCS, antibiotics, and 4% tryptose phosphate broth (Difco). The recombinant virus strains rLACV and rLACVdelNSs were grown and maintained as described previously (6).

RNAi detection. Insect cells were infected with LACV at a multiplicity of infection of 10, and maintained in Leibovitz L-15 maintenance medium (1% fetal bovine serum). At predetermined times postinfection, the cells were harvested, RNA was extracted, and small RNAs were enriched by precipitation of high-molecular-weight species with 5% polyethylene glycol 8000–0.5 M NaCl (26). Low-molecular-weight RNAs were fractionated by 16% polyacrylamide–7 M urea gel electrophoresis, transferred to nylon filters, and hybridized at 42°C with a ³²P-labeled viral RNA sense probe. The S segment mRNA from the same extracts was separated by 1.2% agarose gel electrophoresis, blotted, and hybridized with the same probe.

Plaque assay. Vero cells grown on six-well plates to 90% confluence were inoculated with 10-fold serial dilutions of supernatants from infected cells, diluted in DMEM with 2% FCS. After a 1-h incubation at 37°C, the inoculum was removed, and the cells were overlaid with 3 ml of DMEM with 2% FCS, 0.02% DEAE dextran, and 0.3% agar noble (Difco); cells were incubated for another 72 h at 37°C. Cells were fixed and stained with 1% crystal violet, 3.6% formaldehyde, 1% methanol, and 20% ethanol, and titers were calculated from the plaque numbers according to the dilution.

Plasmid constructs and PCR. DNA plasmids were constructed and propagated using standard techniques. PCR was carried out with AccuPrime Pfx DNA polymerase (for cloning purposes; Invitrogen) or Taq DNA polymerase (for diagnostic purposes and addition of single adenines for TA cloning; Eppendorf). TA cloning was done using a pcDNA3.1/V5-His TOPO TA Expression kit (Invitrogen) according to the manufacturer's instructions. A recombinant Semliki Forest virus (SFV) replicon encoding LACV NSs (SFV-LACV NSs)

equipped with an N-terminal FLAG epitope was generated by PCR amplification and cloning into pSFV-1 (35) using the BamHI restriction site. SFV replicons SFV-Luc (expressing firefly luciferase), SFV-TSWV NSs (expressing the NSs of tomato spotted wilt virus), and SFV-NS1 (expressing the influenza A virus NS1 protein) were kindly provided by Michèle Bouloy (Institute Pasteur, Paris, France) and Marcel Prins (Wageningen University, Wageningen, The Netherlands) (22). The minireplicon plasmid pT7ribo-LACV-cM-GFP encoding the enhanced green fluorescent protein (EGFP) gene flanked by the antigenomic LACV M segment promoter sequences under control of the bacteriophage T7 promoter was constructed by inserting the EGFP coding sequence into the LACV M provector pT7ribo-LACV-cMPro (6), using engineered BpII restriction enzyme sites. The eukaryotic expression plasmid pI.18-HA-LACV-NSs was constructed by ligating a PCR fragment encompassing the LACV NSs sequence with a 5' terminal hemagglutinin (HA) tag into the cloning vector pI.18 (kindly provided by Jim Robertson, National Institute for Biological Standards and Control, Hertfordshire, United Kingdom). The T7 promoter-driven expression constructs pTM-LACV-L and pTM-LACV-N were described previously (5). The firefly luciferase (FF-Luc) reporter plasmid for monitoring IFN- β promoter activation (p-125Luc) was kindly provided by Takashi Fujita, Institute for Virus Research, Kyoto University, Japan (58). The control plasmid pRL-SV40 (Promega) contains the *Renilla* luciferase (REN-Luc) gene under control of the constitutive simian virus 40 (SV40) promoter.

RNAi reporter assay. RNAi against an SFV replicon expressing FF-Luc in insect cells was established as described by Garcia et al. (22). pSFV-1-derived plasmids were linearized with SpeI (SFV-Luc and SFV-NS1) or SapI (SFV-TSWV NSs and SFV-LACV NSs) and in vitro transcribed and capped using SP6 RNA polymerase. Replicon RNA was purified using an RNeasy Mini Kit and resuspended in nuclease-free H₂O. For cotransfection experiments with replicon RNA, 2 × 10⁵ U4.4 cells grown in 24-well dishes were transfected using 125 ng of each RNA (as indicated) and 0.75 μ l of Lipofectamine 2000 (Invitrogen). Transfection mixes were not removed from the supernatant over the course of the experiment, and no cytopathic effect or difference in cell numbers was observed. At 3 days posttransfection, cells were lysed in 50 μ l of passive lysis buffer per well, and luciferase activities were determined as described by the manufacturer (Promega).

In vivo reconstitution and passaging of LACV nucleocapsids. Subconfluent monolayers of BSR-T7/5 cells in six-well dishes were transfected with 0.5 μ g each of plasmid pT7ribo-LAC-cM-GFP, pTM-LACV-L, and pTM-LACV-N using the Fugene 6 transfection reagent (Roche) in 200 μ l of serum free-medium (Opti-MEM; Gibco-BRL). At 24 h posttransfection, cells were infected with viruses at a multiplicity of infection of 5, incubated for 1 h, and washed two times with phosphate-buffered saline (PBS). After an incubation period of 24 h, cells and supernatants containing newly generated viruses were harvested by three freeze-thaw cycles, and centrifuged for 1 min at 14,000 × g to pellet residual cell debris, and 500 μ l was used to infect C6/36 mosquito cells for 1 h. These C6/36 cells had been mock infected or persistently infected with recombinant viruses, as indicated in the text. The superinfected C6/36 cells were washed with medium and then incubated for 24 h. Expression of EGFP and LACV N was assayed by immunofluorescence microscopy as described previously (6).

RT-PCR analyses. Total RNA was extracted from infected cells using the TriFast reagent (Qiagen). For reverse transcription (RT), 1 μ g of total RNA was incubated with 200 U of Superscript II reverse transcriptase (Gibco-BRL) and 100 ng of random hexanucleotides in 20 μ l of 1× RT buffer (Gibco-BRL) supplied with a 1 mM concentration of each deoxynucleotide triphosphate, 20 U of RNasin, and 10 mM dithiothreitol. The resulting cDNA was amplified by 30 cycles of PCR, with each cycle consisting of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C, with a final 10-min step at 72°C. Primer sequences for amplifying mouse β -actin were from Shaw-Jackson and Michiels (46), those for IFN- β were from Marie et al. (36), and those for the LACV N gene were as published previously (6).

Transient transfections and reporter gene assays. Subconfluent cell monolayers grown in six-well dishes were transfected with 0.5 μ g of p125-luc reporter plasmid (FF-Luc gene under control of the IFN- β promoter), 0.05 μ g of control plasmid pRL-SV40 (REN-Luc gene under control of the SV40 promoter), and 1 μ g of expression plasmid in 400 μ l of OptiMEM (Gibco-BRL) containing 5 μ l of poly(I · C) as described previously (55). After overnight incubation, cells were harvested and lysed in 200 μ l of reporter lysis buffer (Promega). An aliquot of 20 μ l of lysate was used to measure FF-Luc and REN-Luc activity as described by the manufacturer of the dual luciferase assay kit (Promega).

Virus growth in tissue culture. MEF cells were grown in six-well dishes to 80% confluence and infected with 0.0001 PFU per cell, washed twice with PBS, and

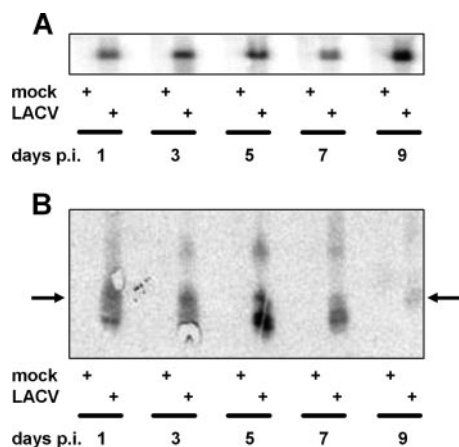


FIG. 1. Formation of LACV-specific small RNAs during persistent infection of C6/36 mosquito cells. Cells were infected with 10 PFU per cell of LACV and passaged twice weekly, and total RNAs were extracted at given time points. (A) To monitor infection, RNAs were separated by 1.2% agarose gel electrophoresis and hybridized to a negative sense S segment RNA probe. (B) Small RNAs were detected after precipitation of the RNA samples with 5% polyethylene glycol, separation on 16% polyacrylamide gels, and hybridization using the same RNA probe as used in panel A. Arrows show the positions of 20-nucleotide markers which were run on the same gel. p.i., postinfection.

supplied with DMEM containing 10% FCS. Supernatants were taken at various times postinfection and assayed in plaque assays on Vero cells.

Pathogenicity studies. Wild-type (wt) C57BL/6 mice and congenic mutant mice with targeted disruptions of the α -subunit of the IFN- α/β receptor (40) or the IFN- β gene (20) were bred locally. Groups of 6 mice at an age of 16 to 18 days were inoculated intraperitoneally with 10,000 PFU of virus in 0.1 ml of PBS. The animals were monitored twice daily over a 9-day period. Mice that were moribund or severely paralyzed were killed.

ISH studies. For in situ detection of IFN mRNAs, RNA probes described in Delhaye et al. (17) were used. The probe used for IFN- α detection was complementary to IFN- $\alpha 5$ (the predominant IFN subtype in the central nervous system) but can recognize other IFN- α subtypes as well. Brain preparation and in situ hybridizations (ISH) were performed as described previously (17). Sections were cut at 8 or 12 μ m. Control hybridizations performed with positive-sense probes instead of antisense probes failed to yield any signal.

For ISH in combination with immunohistochemistry of neurons, sections were treated for immunohistochemistry immediately after the last washes of the hybridization. Sections were blocked in TNB (0.1 M Tris-HCl [pH 7.50] 0.15 M NaCl, 0.5% blocking reagent; Perkin-Elmer) and then incubated with the rabbit anti-LACV N antibody for 2 to 12 h at room temperature. The Dako CSA system (K1500) or Envision kit (K4006/K4010) were used for detection and diaminobenzidine staining.

Immunohistochemistry analysis. Freshly collected brains were immersed in Tissue-Tek optimal cutting temperature compound (Sakura) and frozen at -80°C . Tissue sections 7 μ m thick were cut in a cryostat, placed on SuperFrost Plus slides, and dried at 37°C overnight. Sections were fixed with ice-cold acetone for 10 min and washed before processing for immunohistochemistry. Antibodies used for double labeling were a polyclonal antibody directed against the N protein of LACV and monoclonal antibodies directed against either the neuron-specific nuclear protein ([NeuN] MAB377; Chemicon), the astrocyte-specific glial fibrillary acidic protein ([GFAP] 13-0300; Zymed), the oligodendrocyte-specific myelin basic protein (MAB386; Chemicon), or the microglia/macrophage-specific markers CD11b (553308; Pharmingen) and F4/80 (MCA497R; Serotec). Secondary antibodies were either labeled with Alexa 488 or with Alexa 594 (Molecular probes).

RESULTS

Formation of short LACV-specific RNAs in persistently infected insect cells. Insects do not mount antibody-mediated

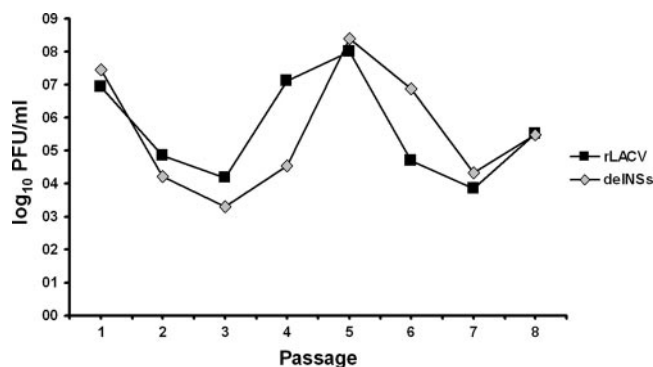


FIG. 2. Time course of persistent LACV infection of mosquito cells. C6/36 cells were infected with 10 PFU per cell of either rLACV or rLACVdelNSs and passaged twice weekly. Viral titers in the supernatants were determined by plaque assay.

immune responses (31). For the defense of viral pathogens, they deploy instead mechanisms like the ubiquitous RNAi system, which is based on the sequence-specific recognition and destruction of viral RNA (44, 54). We wondered whether insect cells are capable of mounting an RNAi response against LACV. To investigate this, we established persistent infection of *A. albopictus* C6/36 cells with LACV and monitored the formation of virus-specific small RNAs, the hallmark of RNAi. Conventional Northern blot analysis of RNAs which were extracted from mock-infected or LACV-infected C6/36 cells at predetermined time points postinfection confirmed that infection with LACV was stable and persistent (Fig. 1A; see also Fig. 2). Fractionation and detection of low-molecular-weight RNAs derived from LACV demonstrated that C6/36 cells indeed produce small RNAs in response to infection (Fig. 1B). We have cloned and sequenced the small RNAs from LACV-infected C6/36 cells and confirmed them to be derived from the LACV S segment (data not shown). Similar observations were obtained in *A. triseriatus* MAT cells (data not shown), indicating a general phenomenon. Of note, microscopic examinations of infected cell cultures did not reveal any signs of cell death (data not shown), as observed previously (7).

NSs does not influence virus growth in insect cells. LACV NSs expressed from a transfected cDNA plasmid was previously shown to inhibit the RNAi pathway in mammalian cells (49). Our newly generated recombinant viruses expressing (rLACV) or lacking (rLACVdelNSs) NSs offer the possibility to evaluate the influence of NSs in the viral context. To determine if the anti-RNAi activity of NSs is important to establish or maintain infection in arthropods, we compared the persistence of rLACV and rLACVdelNSs in insect cells. C6/36 cells were infected with recombinant viruses, cells were passaged over several weeks, and titers in the supernatants were determined at every passage. As shown in Fig. 2, both viruses exhibited the wave-like titer pattern which is typical for growth of bunyaviruses in insect cells (45). No difference was observed between rLACV and rLACVdelNSs, indicating that NSs is not required for infection and persistence of LACV in insect cells.

Protection from superinfection. Insects infected with LACV are resistant to superinfection with California serogroup orthobunyaviruses but remain permissive for Bunyamwera serogroup orthobunyaviruses (1). This pathogen-derived resistance

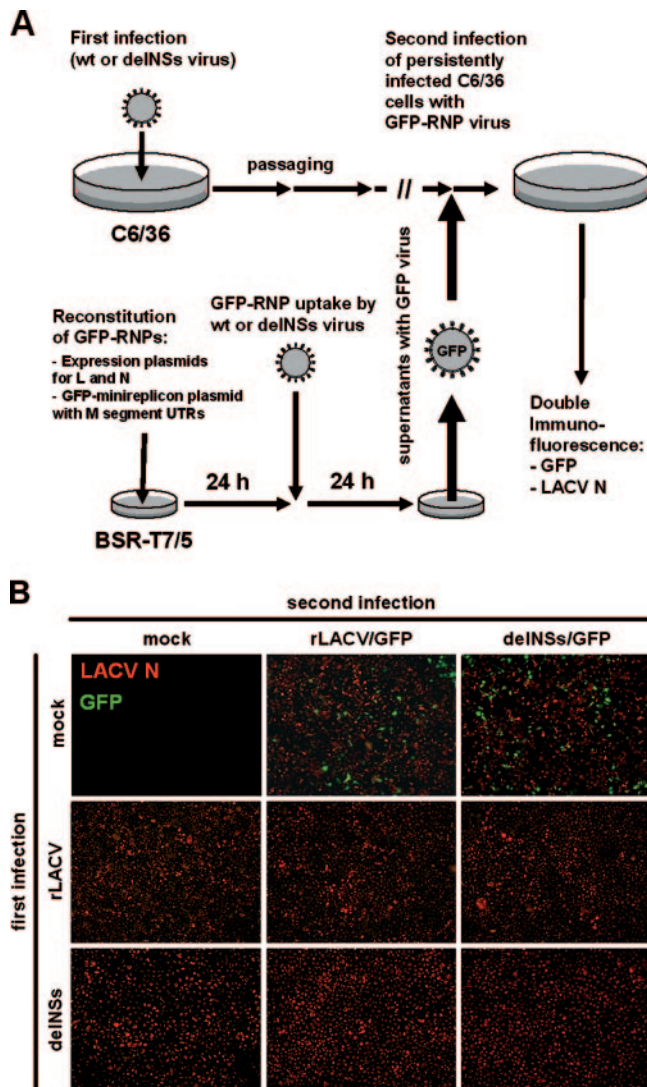


FIG. 3. Protection of C6/36 mosquito cells from superinfection. (A) Experimental outline. C6/36 mosquito cells were infected with LACV and maintained for several passages to establish persistent infection (first infection). The persistently infected cells were then superinfected with GFP-expressing reporter viruses (second infection). The reporter viruses were generated by incorporating GFP-RNPs (reconstituted by transfecting BSR-T7/5 cells with expression constructs for N, L, and a GFP minireplicon) into LACV particles as a fourth segment. Both first and second infections involved either wt viruses or delNSs mutants. (B) Results. C6/36 mosquito cells persistently infected with either rLACV or rLACVdelNSs or left uninfected (mock) were superinfected with GFP reporter viruses. At 24 h postinfection, cells were fixed and stained with antibodies against GFP and LACV N protein.

(PDR) is most likely based on RNAi (4, 21, 44), since expression of the LACV S sequences by recombinant Sindbis virus is sufficient to protect insect cells from infection with LACV (41). Given that LACV provokes an RNAi response in mosquito cells and that transfection of LACV NSs blocks RNAi in mammalian cells, we wondered about the influence of NSs on the PDR phenomenon in insect cells. To investigate this, we superinfected persistently infected insect cells with recombinant

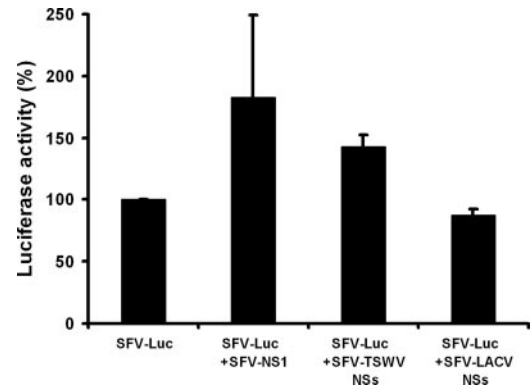


FIG. 4. Effect of LACV NSs on RNAi in insect cells. U4.4 cells were transfected with SFV-Luc, SFV-NS1, SFV-TSWV NSs, or SFV-LACV NSs as indicated. At 3 days posttransfection, cells were lysed, and luciferase activities were determined. Mean values and standard deviations from three independent experiments are shown.

viruses expressing a reporter gene. The role of NSs was addressed by using viruses expressing or lacking NSs in the first, persistent infection, as well as in the second, the superinfection. The outline of the experiment is depicted in Fig. 3A. Insect cells infected with recombinant viruses and passaged to establish persistency were superinfected with GFP-expressing reporter viruses. For the generation of the reporter viruses, we took advantage of an improved version of our LACV RNP reconstitution system which allows efficient incorporation of GFP-expressing reporter gene segments into LACV particles (5). Detection of GFP in superinfected insect cells indicates that the second virus infection was successful, whereas absence of GFP indicates that PDR took place. As expected, mock-infected insect cells were readily infected with GFP-expressing rLACV or rLACVdelNSs (Fig. 3B). However, in persistently infected cells, no GFP expression was observed, indicating establishment of PDR. Importantly, all combinations of rLACV and rLACVdelNSs used for the first infection and/or the superinfection led to the same resistance to superinfection, strongly suggesting that neither the ability of the first viruses to establish PDR nor the inability of the second viruses to overcome it was dependent on NSs.

NSs does not inhibit RNAi in insect cells. We employed an SFV-based reporter assay (22) to test whether NSs would be capable of suppressing RNAi in insect cells. *A. albopictus* U4.4 cells were chosen because C6/36 cells show cytopathic effects after infection with SFV (15). Garcia et al. have demonstrated that FF-Luc expression by an SFV replicon (SFV-Luc) in insect cells is reduced due to RNAi over time but that expression can be rescued by coexpressed RNAi inhibitors (22). U4.4 cells were transfected with SFV-Luc and cultivated for 3 days to establish an RNAi response. Figure 4 shows that cotransfection with SFV-NS1 enhanced luciferase expression by SFV-Luc, as expected (22), since the double-stranded RNA (dsRNA)-binding NS1 can neutralize interference by small RNAs (34). Similarly, the NSs protein of TSWV, a known RNAi inhibitor (9), rescued SFV expression to some extent. By contrast, SFV-LACV NSs had no such enhancing effect, indicating the absence of anti-RNAi activity. Identical results were obtained

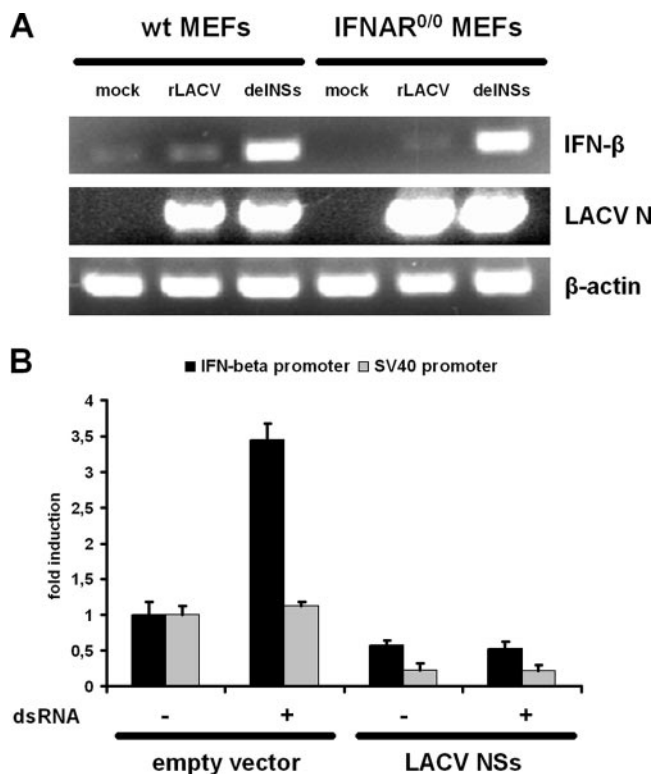


FIG. 5. Effect of LACV NSs on IFN induction. (A) Detection of IFN- β mRNA in infected mammalian cells. MEFs isolated from wt or IFNAR^{0/0} mice were either mock infected or infected with rLACV or rLACVdelNSs. At 18 h postinfection, RNA was extracted and investigated by RT-PCR for the presence of mRNAs for IFN- β , LACV N protein, or β -actin. (B) Suppression of the IFN- β promoter by NSs. Human 293 cells were transfected with luciferase-expressing plasmids under control of either the IFN- β promoter (FF-Luc) or the constitutively active SV40 promoter (REN-Luc), along with the NSs expression plasmid pL18-HA-LACV-NSs or the empty vector. At 8 h post-transfection, cells were either mock treated or transfected with dsRNA and lysed 18 h later to measure luciferase activity. Mean values and standard deviations from four independent experiments are shown.

with recombinant SFV suicide particles infecting *Aedes pseudo-scutellaris* Ap61 cells (data not shown).

Together, our results shown so far imply that the LACV-specific RNAi response mounted in insect cells (Fig. 1) is not countered by NSs, since NSs neither confers a growth advantage (Fig. 2) nor influences PDR (Fig. 3) nor inhibits RNAi against a heterologous virus (Fig. 4). It therefore appears that the anti-RNAi activity of NSs observed in mammalian cells plays no decisive role for LACV in the mosquito vector.

Inhibition of IFN induction in fibroblasts. Previous work demonstrated that NSs proteins of BUNV as well as RVFV efficiently block the antiviral type I IFN response of mammalian hosts (3, 8, 55). To measure this activity for LACV, we infected IFN-competent wt MEFs with recombinant viruses and measured IFN induction by RT-PCR. Figure 5A shows that rLACVdelNSs strongly activated transcription of the IFN- β gene. NSs-expressing rLACV, by contrast, did not induce significant amounts of IFN. Similarly, MEFs derived from mice lacking the type I IFN receptor (IFNAR^{0/0} MEFs) produced IFN- β after infection with rLACVdelNSs, indicating

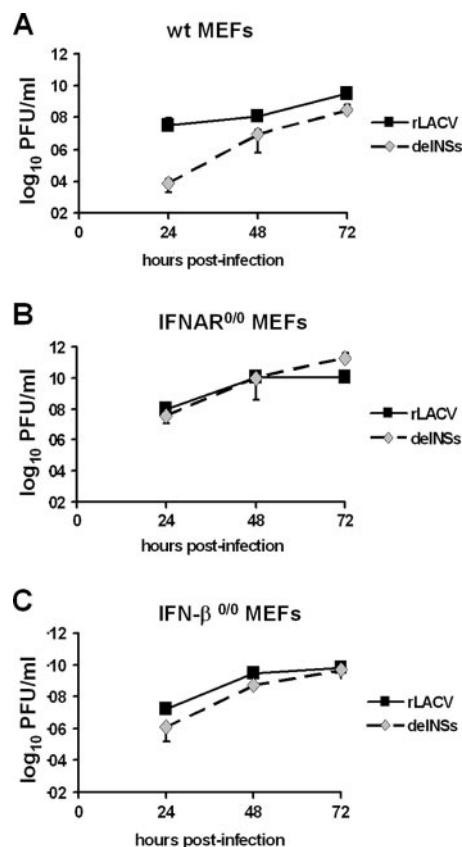


FIG. 6. Growth of viruses in IFN-competent and IFN-deficient cells. Primary MEFs derived from wt mice (A), IFNAR^{0/0} mice (B), or IFN- β ^{0/0} mice (C) were infected with rLACV or rLACVdelNSs at 0.0001 PFU per cell, and virus titers in the supernatants were determined at 24, 48, and 72 h postinfection. Mean values and standard deviations from three independent experiments are shown.

that IFN induction occurred in a direct manner and was independent of secreted IFN. Control RT-PCRs for the LACV N gene showed that viruses replicated to comparable levels, and RT-PCRs for the cellular β -actin mRNA demonstrated that all preparations contained similar amounts of RNA. Of note, RT-PCR analysis for some RNA polymerase II-dependent, constitutively expressed cellular genes shows a strong reduction in wt-infected cells (6), supporting the view that LACV NSs may influence cellular transcription in a manner similar to BUNV NSs and RVFV (32, 33, 51).

To confirm that the block in IFN induction is solely mediated by NSs, we measured its effect on IFN- β promoter activity independent of the viral context. Human 293 cells were transfected with an NSs-expressing plasmid and an IFN- β promoter reporter plasmid, and promoter activity was measured after stimulation of cells with synthetic dsRNA. As shown in Fig. 5B, expression of LACV NSs efficiently inhibits induction of the IFN- β promoter by dsRNA, confirming that NSs is sufficient to suppress IFN production.

Effect of NSs on viral growth in IFN-competent and -deficient systems. Given the strong effects of LACV NSs in mammalian cells on apoptosis (6, 14), RNAi (49), and IFN induction (Fig. 5), it was of interest to us to clarify which one of these activities is relevant to the virus multiplication in the

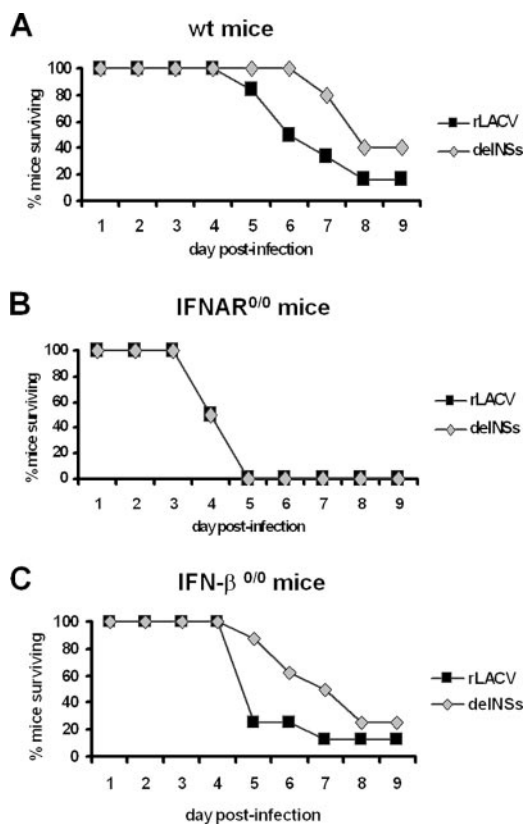


FIG. 7. IFN-dependent virulence of viruses in vivo. Survival of wt (A), IFNAR^{0/0} (B), or IFN-β^{0/0} mice (C) infected with 10,000 PFU of rLACV or rLACVdelNSs was monitored. Mice that were moribund or severely paralyzed were killed and scored dead on that day.

mammalian host. Cells lacking an IFN system are still able to undergo apoptosis induced by NSs (6), and in mammalian cells the antiviral IFN system dominates the RNAi system (18, 47). We therefore expected that an IFN-independent influence of NSs on virus replication would show up in IFN-deficient cells or animals. First, we compared the multistep growth of rLACV and rLACVdelNSs in primary MEFs derived from wt and IFNAR^{0/0} mice. Since the delNSs mutant strongly activates IFN-β transcription (Fig. 5A), we also investigated the role of IFN-β by using cells from mice with a deleted IFN-β gene (IFN-β^{0/0} MEFs). Figure 6A shows that in IFN-competent wt MEFs, the NSs-expressing rLACV had a clear growth advantage over the delNSs mutant. A difference of 4 log steps was observed at 24 h postinfection. Although this gap was reduced to a difference of about 1 log step at 72 h postinfection, rLACV was always superior to rLACVdelNSs. By contrast, in IFNAR^{0/0} MEFs which are unable to respond to IFN, no apparent difference was observed at 24 h postinfection, and at 72 h postinfection the titers of rLACVdelNSs were even higher than those of rLACV (Fig. 6B). In IFN-β^{0/0} MEFs, the delNSs mutant multiplied at a slightly lower rate than rLACV, but the difference between the two viruses was much less pronounced than in wt MEFs. This indicates that IFN-β contributes to the growth restriction of rLACVdelNSs in cell culture.

We also investigated the interaction of LACV NSs and the IFN system in vivo. wt and mutant mice were inoculated with

TABLE 1. Virus growth in brains of diseased IFNAR^{0/0} mice

Virus	Animal no.	PFU/brain ^a
rLACV	1	8 × 10 ⁷
	2	4 × 10 ⁷
	3	6 × 10 ⁷
rLACVdelNSs	1	6 × 10 ⁷
	2	6 × 10 ⁷
	3	4 × 10 ⁷

^a Brain weight was 0.4 g on average.

the recombinant viruses by the intraperitoneal route to mimic natural infection by mosquitoes (48). The NSs-expressing rLACV killed IFN-competent wt mice more rapidly and more efficiently than the delNSs mutant (Fig. 7A). IFNAR^{0/0} mice, by contrast, were killed by both viruses with the same efficiency (Fig. 7B), and both viruses reached comparable titers in the brains of diseased animals (Table 1). IFN-β^{0/0} mice displayed

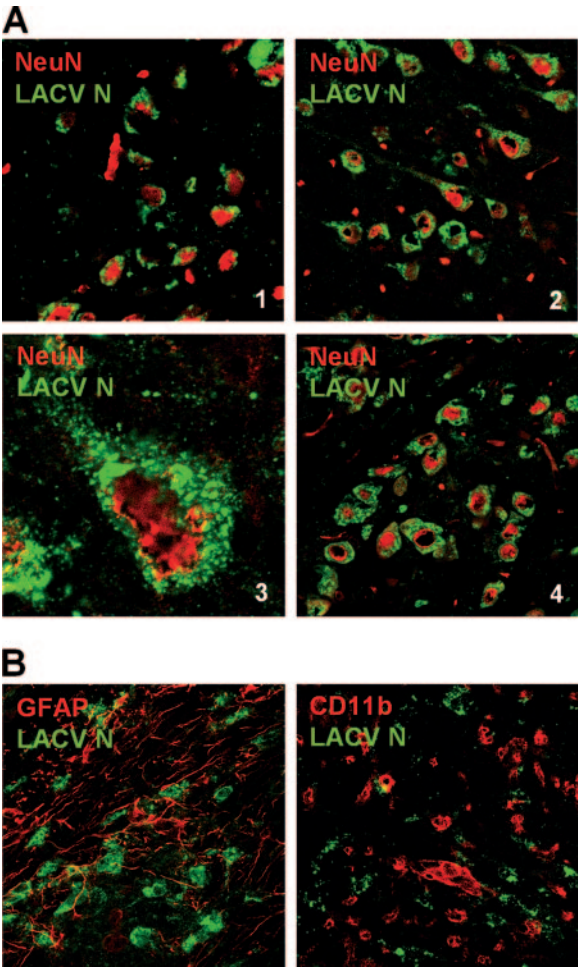


FIG. 8. Infection of neurons in vivo. (A) Brain sections of wt mice infected with rLACV (frame 1) or rLACVdelNSs (frames 2 to 4) were stained for viral antigen (green) and the neuron marker NeuN (red). Frames 1 to 3 show sections of the cortex, and frame 3 is a magnification of the section shown in frame 2. Frame 4 shows a section of the thalamus. (B) Brain sections from mice infected with rLACVdelNSs were double stained for viral antigen and markers for astrocytes (GFAP) or macrophages (CD11b).

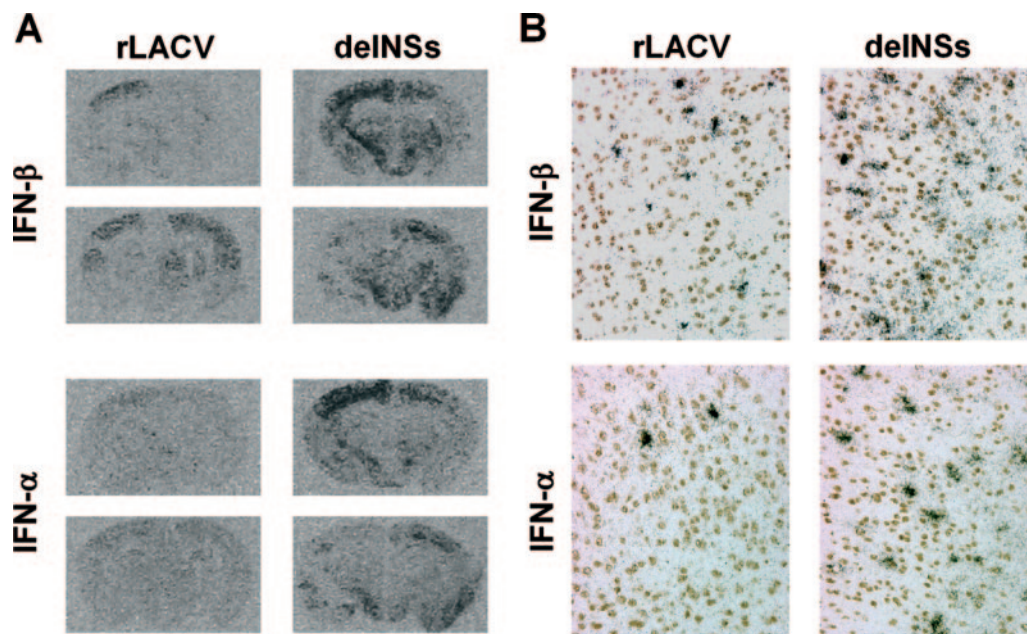


FIG. 9. Induction of IFN in the mouse brain. ISH with IFN- β or IFN- α probes (see Materials and Methods) was performed on brain sections of mice infected with rLACV or rLACVdelNSs. Macroscopic analysis (A) and microscopic analysis (B) using a combination of ISH to detect IFN- β or IFN- α mRNA (black silver grains) and immunohistochemical analysis for LACV N (brown stain).

an intermediate type of survival kinetics (Fig. 7C), as these animals succumbed more rapidly to infection than their wt counterparts but more slowly than the IFNAR^{0/0} mice. This suggests that IFN- β plays a minor, but not a decisive, role in the protection from LACV infection in vivo.

Infection of neuronal cells. In the mammalian host, including humans, LACV is known to disseminate into the central nervous system and infect neuronal cells (28), causing severe aseptic encephalitis (23). To investigate whether rLACVdelNSs preferentially infects neuronal cells in a manner similar to rLACV, we analyzed the brains of wt mice with severe disease. In situ double immunofluorescence was performed using a rabbit polyclonal antibody directed against the LACV N protein together with various mouse monoclonal antibodies for markers of major brain cell types. NeuN-positive cells were heavily infected by both rLACV and rLACVdelNSs (Fig. 8A, frames 1 to 3), indicating that both viruses preferentially infected neurons. In some areas of the thalamus, almost 100% of the neurons were infected (Fig. 8A, frame 4). By contrast, in cells positive for the astrocyte marker GFAP, the oligodendrocyte marker myelin basic protein, or the macrophage markers CD11b and F4/80, no viral antigen was clearly detected (Fig. 8B and data not shown). Thus, rLACVdelNSs has the ability to disseminate in the brain and to preferentially infect neurons.

Induction of IFN by neurons. Given the similar ability of LACV possessing or lacking NSs to infect neurons, we wondered whether LACV would also block IFN induction in the central nervous system. To address this question, we compared IFN synthesis in brains from mice infected with either rLACV or rLACVdelNSs. ISH analysis of whole-brain slices demonstrated that rLACVdelNSs induced significantly higher IFN- β and IFN- α transcript levels than rLACV (Fig. 9A). To corre-

late in vivo IFN mRNA synthesis with virus infection, we combined the analysis of IFN mRNA with immunostaining against the LACV N protein. Examination of highly infected brain areas showed that brain cells infected with the delNSs mutant were more frequently positive for IFN mRNAs than those infected with rLACV (Fig. 9B). We therefore concluded that the IFN antagonist NSs of LACV is also functional in neurons.

DISCUSSION

In this study, we provided in vivo evidence that the IFN antagonism of LACV NSs constitutes its major biological function. In cells as well as in animals infected with wt rLACV, synthesis of antiviral type I IFN mRNAs was strongly impaired, demonstrating that LACV NSs abrogates IFN induction. Consequently, wt rLACV had a growth advantage over the delNSs mutant in IFN-competent cells and mice. By contrast, in all IFN-deficient systems including insect cells, no apparent difference was observed between the two viruses. Thus, NSs is only important in IFN-competent mammalian hosts and the deletion of the viral NSs gene can be fully complemented by inactivation of the host's IFN system. This strongly implies that bunyaviruses, which as arboviruses need to rapidly establish viremia in order to be transferred to new insect vectors, evolved the accessory NSs gene to overcome the fast-responding innate immune defense of the mammalian host.

Despite the strong effects of NSs on apoptosis (6, 14), no detectable differences in growth and virulence of our virus pair exist in IFN-deficient mammalian host systems (Fig. 6 and 7). This suggests that the proapoptotic function of LACV NSs has no apparent consequences for virus growth or virulence in vivo. The ability to promote (as well as to prevent) apoptosis was demonstrated for many different viruses (2), and it is thought

that the correct timing of cell death is important to optimize virus yields. However, reports have been contradictory with respect to the biological significance of virus-induced apoptosis. For influenza A virus, which strongly promotes apoptosis (27), reverse genetics experiments revealed no correlation between the levels of apoptosis in infected cells and the extent of virus replication (39). In line with this notion, it was found that BUNV NSs has the opposite activity of LACV NSs. It inhibits rather than promotes apoptosis (29), which raises a question about the role of programmed cell death in the bunyavirus infection cycle. The proapoptotic function of LACV NSs was shown in mammalian cells, and its importance for virus replication was inferred from its similarity with a C-terminal part of the proapoptotic *Drosophila* protein reaper (14). However, given the fact that reaper is an insect protein, it is curious that NSs-expressing LACV as well as other bunyaviruses do not induce cell death in insect cells (7). Thus, despite the sequence similarity to the proapoptotic *Drosophila* reaper, NSs has no apparent proapoptotic activity in insects, suggesting that NSs is not simply a reaper protein that was snatched by the bunyaviruses to promote cell death of infected cells. Rather, we favor the assumption that the proapoptotic function of LACV NSs is a side effect of its molecular mechanism to block IFN induction.

Expression of NSs from a cDNA construct was shown to counteract the RNAi effect of transfected RNA oligonucleotides (49). RNAi is regarded as an antiviral mechanism of insects (44), and we show that LACV infection provokes a strong RNAi response in insect cells (Fig. 1). In mammalian cells RNAi effects can be observed if the IFN system is deleted (47) or circumvented by using short dsRNAs to induce RNAi (18). Using recombinant viruses, we addressed the supposed role of NSs in RNAi inhibition both in the mosquito system and the mammalian host. Surprisingly, we could not detect any advantage conferred by NSs expression in insect cells (Fig. 2) or in IFN-deficient mammalian cells and animals (Fig. 6 and 7). Also, in insect cells the NSs-expressing rLACV virus established PDR to the same extent as the NSs-deleted mutant did, and both viruses were equally subject to PDR (Fig. 3). Furthermore, LACV NSs was unable to counteract RNAi in insect cells (Fig. 4), just as was shown for RVFV NSs (22). Similarly, the NSs of BUNV has no effect on viral transcription in insect cells (A., Kohl and R. M. Elliott, presented at the 12th International Conference on Negative-Strand Viruses, Pisa, Italy, 14 to 19 June 2003). These data do not support the view that bunyavirus NSs plays a role as an anti-RNAi factor in the context of infection. A similar negative conclusion was recently drawn for other viral proteins with a proposed anti-RNAi function (16, 30).

Our *in vivo* analyses not only established NSs as the IFN antagonist of LACV but also demonstrated that both IFN- β and IFN- α contribute to protection from LACV infection (Fig. 6 to 9). Nevertheless, a high percentage of wt mice eventually succumbed to infection with LACV, even when the strong IFN-inducer rLACVdelNSs was used (Fig. 7). It should be noted that the most effective IFN-induced protein with antiviral activity against LACV is the human MxA protein (24) and that the gene for the murine homologue Mx2 is defective in most inbred mouse strains including the C57BL/6 mice used in our experiments (50). We therefore assumed that our mice

could not launch a pronounced antiviral response against rLACVdelNSs because they lack an Mx protein with anti-bunyaviral activity. To follow this up, we compared the antiviral effect of type I IFN against wt and delNSs virus in the MxA-expressing human Huh7 cells and the Mx-negative mouse 3T3 cells. IFN inhibited both viruses much more strongly (2 log steps) in the human cell line than in the mouse cell line (data not shown). Thus, the weak attenuation of the delNSs virus in wt mice is, indeed, most probably due to the lack of the suitable IFN effector protein.

In summary, we present evidence that the major biological function of LACV NSs is to inhibit the antiviral type I IFN system in the mammalian host. Further investigations of the molecular mechanism of NSs action might reveal an interesting link between apoptosis induction, the RNAi system, and the IFN system.

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